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Aldehyde Fixation Differentially Affects Distribution of Diaphorase Activity But Not of Nitric Oxide Synthase Immunoreactivity in Rat Brain

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ABSTRACT: The effect of aldehyde fixation on NADPH- and NADH-dependent diaphorase (d) histochemistry and nitric oxide synthase (NOS) immunocytochemistry in the brain was investigated by comparing the distribution of these enzymes in in situ nitrocellulose blots of unfixed brain sections with that in aldehyde-fixed brain sections. Substitution of NADPH by NADH yielded no gross differences in cellular distribution in the native blot, whereas in fixed sections NADH produced nonspecific staining of the entire section. In the in situ blot NADPHd histochemistry therefore visualized general nitroblue tetrazolium reductase (NBTr) activity, which was particularly strong in hippocampal pyramidal neurons and cerebellar Purkinje cells. Aldehyde fixation abolished the anatomical pattern of general NBTr activity and changed the histochemical distribution in that of the NADPHd activity associated with the distribution of NOS-I immunoreactivity (ir). Fixation intensified NADPHd histochemical staining in specific neurons, resulting in outstanding, Golgi-like staining of these neurons in several brain regions, whereas the general NBTr activity in pyramidal and Purkinje cells disappeared. In contrast to the histochemical diaphorase distribution, the distribution of NOS-I ir on blots and in aldehyde-fixed brain sections was similar. No NOS was observed in hippocampal pyramidal and cerebellar Purkinje neurons. In regions like cerebral and cerebellar cortex and striatum the applied anti NOS-I serum had a higher affinity for the native protein. It is concluded that aldehydes, rather than to progressively suppress NOS-unrelated enzymes, differentially elicit NADPHd activity in some groups of neurons while leaving NOS-ir unaffected.

KEY WORDS: NOS, NADPH, NADH, Protein in situ blotting, Enzyme activity detection, Neuroanatomy, Hippocampus, Cerebellum.

INTRODUCTION

In the aldehyde-fixed brain, NADPH diaphorase (NADPHd) activity, that is, the NADPH-dependent conversion of nitroblue tetrazolium (NBT) to blue NBT diformazan, has been demonstrated histochemically in discrete neuronal populations, forming a unique pattern that is distinct from any other neurochemically defined cell group [18,22,24]. Recently it was reported that neuronal NADPHd is identical with type I nitric oxide (NO) synthase (NOS-I) [6,11]. This Ca^{2+}/calmodulin dependent enzyme catalyzes the NADPH-dependent formation of NO and citrulline from the precursor amino acid L-arginine [4,15]. NO is a neuronal messenger [3,9,10] proposed to play a role in synaptic plasticity [7,20,21]. Because neuronal NADPHd and NOS are identical in brain and peripheral tissues [6,11], NADPHd histochemistry is frequently applied to localize NO releasing cells in the central and peripheral nervous system. However, several other enzymes, for example, cytochrome C and cytochrome P450 reductase in the brain, also display NADPHd activity [8,12–14] and, in crude supernatant fractions of brain homogenates, a correlation between NOS and NADPHd can only be demonstrated after treatment with aldehyde fixation [16]. All neuroanatomical studies addressing localization and distribution of NADPHd are performed on aldehyde-fixed brains sections. However, aldehydes cause protein linkage that may affect enzyme activity and thereby influence the histochemical properties of proteins. Aldehyde bonds further can hinder binding properties of antibodies to their epitope. In these ways tissue fixation may cause methodological errors in localizing NOS-I immunoreactivity (ir) or NADPHd activity. Therefore, the distribution of histochemical NADPHd and NOS-ir under aldehyde-fixed and unfixed conditions was compared. This is of particular interest because functional and morphological studies in hippocampus and cerebellum disagree on the location of the source of NO release [7,20,22].

Recently, we described a procedure for the direct transfer of proteins from frozen native tissue sections to an immobilizing matrix, that is, nitrocellulose membrane, thereby avoiding tissue fixation and providing optimal accessibility for protein detection with preservation of the anatomical location [17], which essentially confirms a similar approach for the quantification of autoradiography [25]. The aim of this study was to apply this so-called in situ blotting technique to investigate the distribution of NADPHd and NOS in unfixed brain tissue and study the impact of aldehyde fixation on the distribution of these enzymes.

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MATERIALS AND METHODS

Chemicals

Tris, β-nicotinamide adenine dinucleotide (phosphate), reduced form (NAD(P)H), nitroblue tetrazolium (NBT), and 4-chloro-1-naphtol were obtained from Sigma (St. Louis, MO). Nitrocellulose (NC) membranes with a pore size of 0.45 μm (Schleicher & Schuell, BA85, Germany) were used for in situ blotting. The following immunochemicals were applied for detection of NOS in sections and blots: rabbit polyclonal anti-NOS-I (bleeding number 6763-5; ref. 19); biotinylated goat anti-rabbit IgG (*F*(ab') fraction, Zymed, San Fransisco, CA); and streptavidin-horseradish peroxidase (HRP) (Zymed). Buffers frequently used during histochemical and immunocytochemical procedures were: 0.1 M phosphate buffer (PB) (pH 7.4); 0.05 M Tris-HCl buffer (TB) (pH 7.6); 0.04 M Tris buffered saline (TBS) (0.9% NaCl, pH 7.4); TBS with 0.05% Triton X-100 (TTBS, pH 7.4).

Animals and Tissue Preparation

All procedures were carried out on Wistar rats according to the requirements of the National Act on the Use of Experimental Animals (The Netherlands). Male rats of 250–350 g body weight were transcardially perfused with TBS (500 ml/kg) after an overdose of pentobarbital anesthesia (60 mg/kg IP). For native in situ blotting, brains were quickly removed, frozen with liquid nitrogen, and cut with a cryostat microtome at −12°C (thickness of 20 μm). To study the enzyme distribution in aldehyde-fixed brain, the animals were transcardially perfused with PBS followed by 300 ml of a relatively mild fixative composed of 2% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in PB at a perfusion speed of 20 ml/min. Aldehyde-fixed brains were cryoprotected overnight at 4°C in 30% sucrose in PB and subsequently sectioned on a cryostat microtome at −20°C. Part of the mildly fixed sections were postfixed in 2.5% glutaraldehyde in PB to study the effect of very strong aldehyde fixation on immuno- and histochemistry.

Blotting Procedure

Brain sections were blotted on membranes with a slightly modified in situ blotting procedure [17]. In short, the sections (20 μm) were mounted onto dry nitrocellulose (NC) membranes in the cryostat box and quickly thawed by overlay on the back of the hand. Rapid and short thawing facilitates the stretching of sections on the NC membrane. Next, the NC membranes were incubated for 15 min in a humid chamber on filter paper that was prewetted with incubation buffer (0.04 M Tris-HCl, 10%-methanol, pH 7.6). During this incubation the transfer of tissue proteins onto the membrane matrix was achieved by permeation of the cellular content directly from the tissue into the underlying matrix. After protein transfer, the remaining brain tissue was removed from the blotting membrane by a high pressure jet spray with the incubation buffer.

Histochemical and Immunocytochemical Protein Detection in NC Membrane

After removal of brain tissue, NC membranes were soaked in blocking buffer (5% gelatin-0.04 M Tris-HCl, 0.9% NaCl, pH 7.6) for 30 min to prevent nonspecific binding of antibody or substrate to free membrane sites. Then membranes were rinsed for 10 min in TTBS.

The NADPHd activity on the NC membranes was determined by incubating the membranes in TB containing 0.01% NBT and 0.1% β-NADPH for about 1–2 h at room temperature (RT).

The influence of aldehyde fixation on the blotting procedure for NADPHd histochemistry and NOS ir was assessed by blotting aldehyde-fixed (2% paraformaldehyde; 0.05% glutaraldehyde) brain sections and subsequent staining of these blots for NADPHd. Previous fixation of the tissue apparently prevented the "release" of proteins from tissue to blotting membrane because no visible staining appeared with both methods. Furthermore, the effect of postfixation of blotted native proteins was studied. NC membranes after transfer of native proteins were fixed for 5 min with very mild and strong fixative (1% and 4% paraformaldehyde, respectively) followed by NADPHd staining and NOS ir. Mild fixation hardly affected the pattern and density of histo- and immunocytochemical staining. After strong aldehyde fixation both stainings were faint, but patterns were unaffected.

A control assay for the histochemical procedure was performed by either omitting the electron donor substrate NADPH or by substituting it by NADH. Without an electron donor, histochemical staining was absent. Substitution by NADH detects NADH diaphorases or DT diaphorases (NAD(P)H dehydrogenase (quinone)), which can utilize either NADH or NADPH as a cofactor for the reduction of NBT.

For immunocytochemical detection of NOS in blotted brain sections, the NC membranes were incubated overnight with rabbit anti-NOS-I (1:100–1:400) in TTBS containing 1% gelatin. The membranes were subsequently washed with TTBS (three times for 10 min each), reacted with the biotinylated goat anti-rabbit IgG (1:500) for 2 h, and rinsed again. The membranes were then exposed to streptavidin-HRP (1:500) for 1 h. After the final washing the immunoprecipitates were visualized with the HRP substrate 4-chloronaphtol (in TBS containing 3 mM 4ClN, 15% methanol, 0.015% H_2O_2, pH 7.4) until a clear staining pattern occurred. All immunocytochemical procedures were carried out at RT.

Control experiments for immunostaining were performed by omitting the primary antibody, yielding negative results, and by using another primary antibody raised against cerebellar NOS-I (generous gift from Dr. T. M. Dawson, Johns Hopkins Univ., Baltimore, MD), yielding results identical to anti NOS-I used in this study (data not shown). An additional control was performed by adding purified NOS-I to the antibody solution (30 μg/ml anti NOS-I (1:100), resulting in strongly reduced immunostaining. After immunoprocessing, NC membranes were rinsed and stored in distilled water. For light microscopical examination, NC blots were coverslipped in distilled water.

Histochemical and Immunocytochemical Protein Detection in Aldehyde Perfusion Fixed Brain

Histochemical detection of NADPHd in sections was carried out, as for NC membranes, according to earlier described methods [24]. Free-floating sections were incubated in PB, containing 0.3% Triton X-100, 0.1% β-NADPH, and 0.01% NBT at RT for 30–60 min. In the absence of NADPH, no stained cells were detected. To study the effect of aldehyde concentration on the NADPHd activity in brain sections, part of the sections were further postfixed for 2 h in 2.5% glutaraldehyde in PB.

Immunostaining free-floating brain sections for NOS was similar, as described for the immunoblotting procedure. Briefly, after thorough rinsing in phosphate-buffered saline (PBS; 0.01 M, pH 7.4), the sections were immersed in 0.01% H_2O_2 for 15 min and incubated in 5% normal goat serum (NGS, Zymed) at

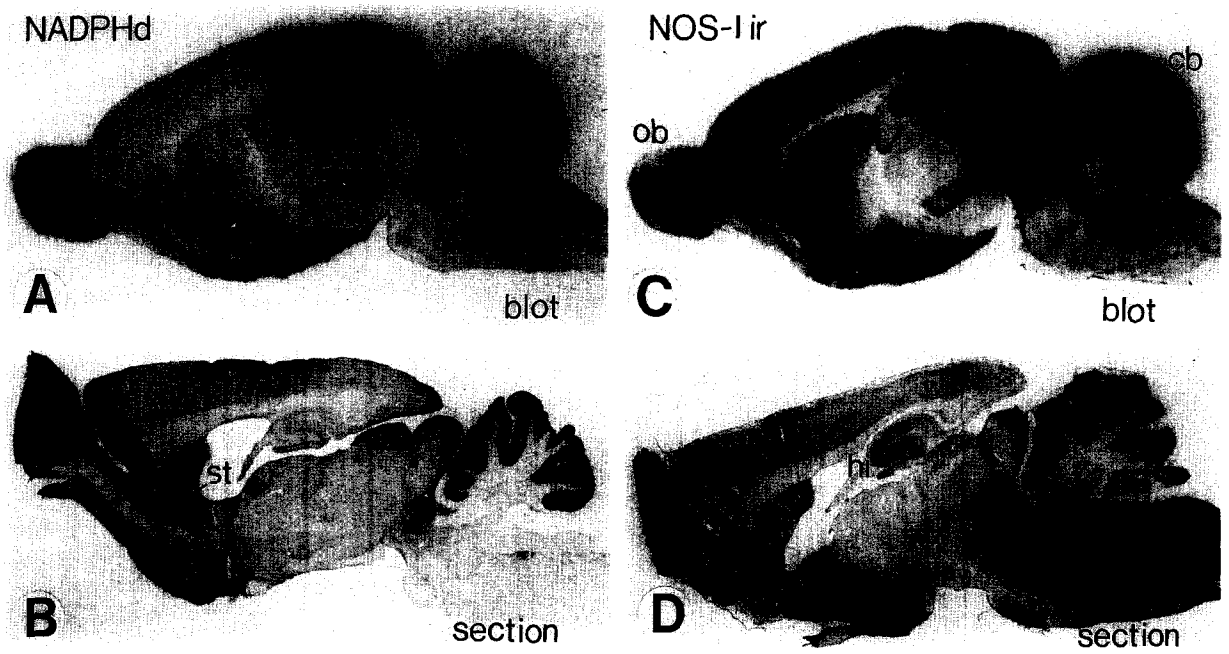

FIG. 1. Photomicrographs showing histochemical NADPHd and immunocytochemical NOS-I immunoreactive (NOS-I ir) staining in sagittal sections of the adult rat brain. (A) and (C) Distributions in native NC blots. (B) and (D) Aldehyde-fixed brain sections. (A) Note dense precipitation of NADPHd in native in-situ blot in hippocampal pyramidal cells, amygdalar regions and olfactory bulb. Clearly visible are also stained Purkinje cells in cerebellum. (B) Conversely, NADPHd histochemical distribution in mildly aldehyde-fixed brain section is strongly positive in cerebellar cortex. Purkinje cells are unstained and visible as a white line. Precipitation is further present in tectum and olfactory bulb. Individual cell staining is strong in cortex and striatum. Note very intense staining in accessory olfactory bulb. (C) Distribution of NOS-I ir in native, blotted brain section showing very dense precipitation in cerebellar cortex, tectum, medial amygdalar regions, and olfactory bulb. Interneurons in cerebral cortex and striatum are densely stained and identifiable as individual dots. In hippocampus, pyramidal cells and CA radiate layer are faintly immunopositive. (D) Distribution of NOS-I in mildly aldehyde-fixed brain section. Staining is dense in cerebellar cortex, hypothalamic, and olfactory regions. Unlike in nitrocellulose blots it is not possible to distinguish individual interneurons in cerebral cortex and striatum at a macroscopical level, although they are distinctly immunopositive in microscopic analysis. Abbreviations: cx, cerebral cortex; cb, cerebellum; hi, hippocampus; ob, olfactory bulb; st, striatum.

RT prior to the first antibody application. Following overnight incubation in rabbit anti-NOS-I (1:100 in PBS containing 1% NGS, 4°C), the sections were incubated in biotinylated goat anti-rabbit IgG (*F*(ab')2 fraction; 1:200; 4 h at RT), and thereafter in streptavidin-peroxidase (1:200, 2 h at RT). Between all incubation steps, sections were rinsed at least three times for 15 min in PBS. Tissue-bound peroxidase was visualized using the diaminobenzidine (DAB) reaction (30 mg DAB in 100 ml TB and 0.01% H_2O_2).

Control experiments for immunostaining of fixed sections were performed as described above.

Histochemical Protein Detection in Mounted Fresh Brain Sections With and Without Aldehyde Postfixation

An additional control for the effect of aldehyde fixation on the histochemical detection of NADPHd and NADHd was provided by mounting unfixed brain sections (10 and 20 μm). Part of these sections were postfixed for 10 min in increasing concentrations of paraformaldehyde: 0.5, 1, 2, and 4% in PB. Subsequently the sections were rinsed in PBS, and enzyme activity was visualized as mentioned above. This additional experiment further substantiated the reliability and sensitivity of the in situ blotting technique.

RESULTS

NADPH Diaphorase Activity

Distribution in NC blots. Substituting NADH for NADPH resulted anatomically in a nearly similar staining pattern, indicating that with the applied histochemical procedure it is not possible to separate and identify NADPHd, NADHd, or DT diaphorase in native blots. Therefore, the observed NBT reductase activity is general and nonselective and has to be partly ascribed to DT diaphorase.

Light microscopic study of the native in situ blots showed a pattern of cellular NBT formazan staining in a large proportion of the neuronal cell bodies and occasionally parts of dendrites throughout the brain (Figs. 1A, 2C,F). In fiber systems like the corpus callosum and the optic tract, also NADPHd positive cells were observed indicative for glial staining.

In the cerebral cortex precipitation was found to be present in the cytoplasm of the neuronal cell bodies occasionally extending into apical dendrites. The pyramidal cells of the hippocampal cornu Ammonis fields CA1–CA4 revealed distinct staining (Fig. 1A), with the highest level of enzyme activity in the CA1 cell layer (Fig. 2C). The stratum radiatum showed a moderate staining mainly caused by precipitation in pyramidal apical dendrites, whereas the stratum lacunosum/moleculare and the outer two-thirds of the molecular layer of the dentate gyrus showed only a weak reaction product. Throughout all hippocampal layers interneurons were faintly visible. The enzyme activity was moderate in the inner one-third of the molecular layer and the granule cells of the dentate gyrus. NBT reductase activity was also evident in cerebellar Purkinje cells and in the molecular layer of the cerebellum (Figs. 1A, 2F). The reaction level was lower in the granule cell layer and in the white matter. Enzyme activity was also notably rich in the substantia nigra pars compacta, the supraoptic

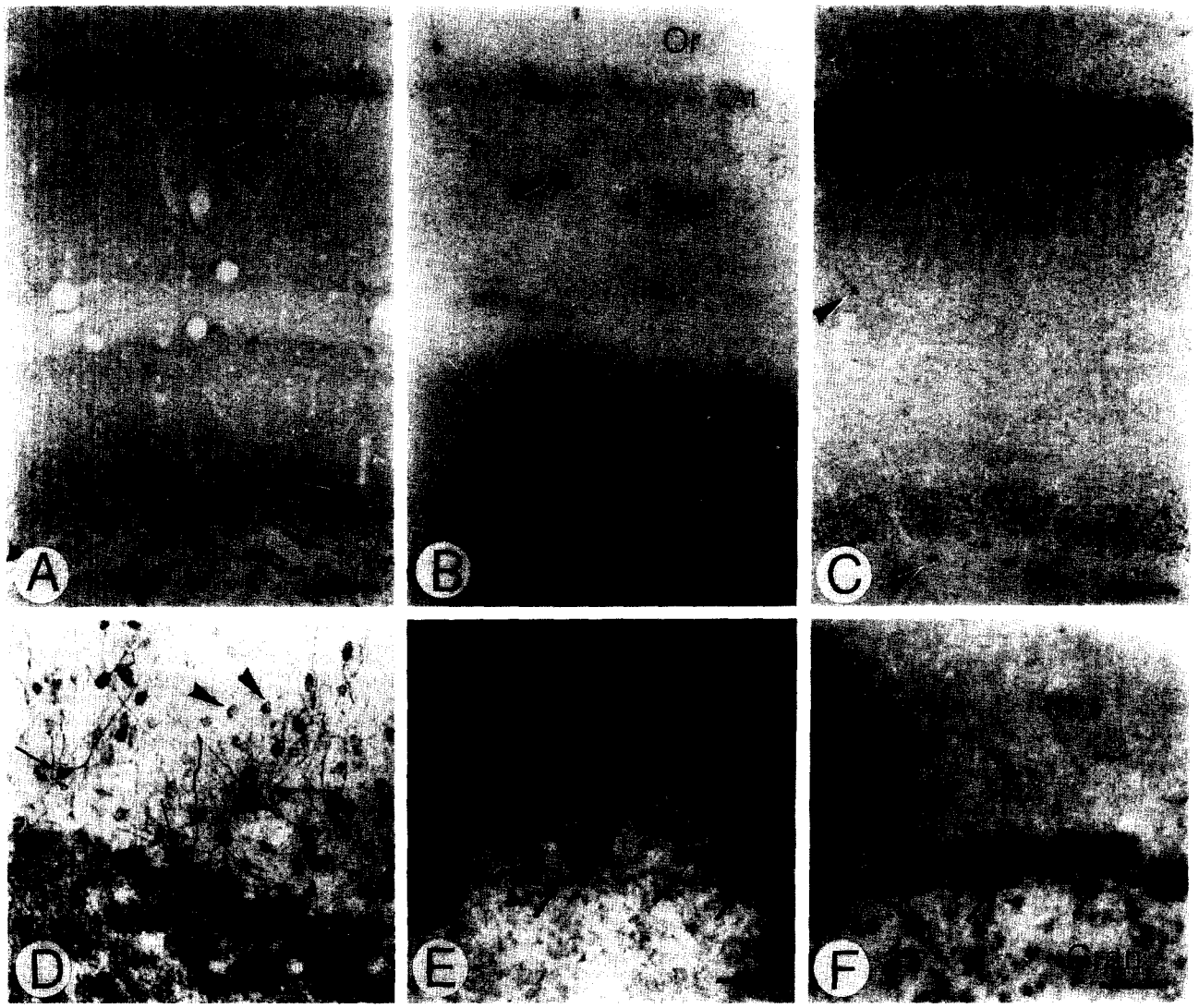

FIG. 2. Photomicrographs of hippocampus (A–C) and cerebellum (D–F) showing localization of NOS-I ir (A, B, D, E) and NADPHd (C–F) in native NC blots (B, C, E, F) and mildly aldehyde-fixed brain sections (A, D). In the hippocampus in fixed brain sections (A) and in NC blot (B) densely stained NOS-I immunopositive interneurons (arrowheads) are visible, whereas pyramidal neurons in both procedures are only very faintly immunopositive. (C) Conversely, hippocampal NADPHd histochemistry in NC blot shows dense staining in pyramidal cells and dendrites of CA1 with faint staining in interneurons (arrowheads). (D) NOS-I ir in aldehyde-fixed cerebellar cortex. Basket cells (small arrows) in molecular layer are densely immunopositive. Arrowheads indicate weaker stained cells with the appearance of stellate cells. Purkinje cells (thick arrows) are faintly immunoreactive. (E) NOS-I ir in NC blot shows faint Purkinje cells (arrows). Basket cells are further detectable (arrowheads). (F) NC blot showing NADPHd in cerebellar Purkinje cells (arrows). Bars: A, B, C = 100 μm; D, E, F = 50 μm. Abreviations: cx, cerebral cortex; cb, cerebellum; hi, hippocampus; st, striatum; ob, olfactory bulb.

nuclei (SON), the mitral cells, and the external plexiform layer in the olfactory bulb, the magnocellular basal nuclei, and the medial septal-diagonal band complex. Dense precipitates were further found in the pineal gland, colliculus superior layers, and striatum. The paraventricular nuclei of the hypothalamus showed a moderate staining of neurons.

Distribution in aldehyde perfusion fixed brain. The distribution of NADPHd activity in aldehyde-fixed brain tissue was in line with that described in other articles [18,24]. Major differences after aldehyde fixation in enzyme activity as compared to the distribution in native blots were revealed in cell groups like the hippocampal pyramidal neurons and interneurons and the cerebellar Purkinje cells. Diaphorase activity in native blotted brain sections was present in both hippocampal and cerebellar cell types with similar staining densities (Figs. 1A, 2C,F). In the mildly fixed brain sections pyramidal neurons in hippocampal CA1–4 showed a faint but clear histochemical reaction (Fig. 3A). Individual interneurons, as indicated by the arrow in Fig. 3A, showed outstandingly intense staining. Interestingly, these densely stained neurons were not visible in the native blots. Cerebellar Purkinje cells were completely negative after mild fixation, whereas molecular and granular layers showed high enzyme activity (Fig. 1B). Increasing the level of aldehyde fixation by immersing free-floating mildly fixed sections in 2.5% glutaraldehyde in PB eliminated all staining in hippocampal pyramidal neurons (Fig. 3B), but did not affect the intensity of the staining of interneurons either in the hippocampal principal cell layer (Fig. 3B, arrow) or in the dendritic layers of CA1–4 and dentate

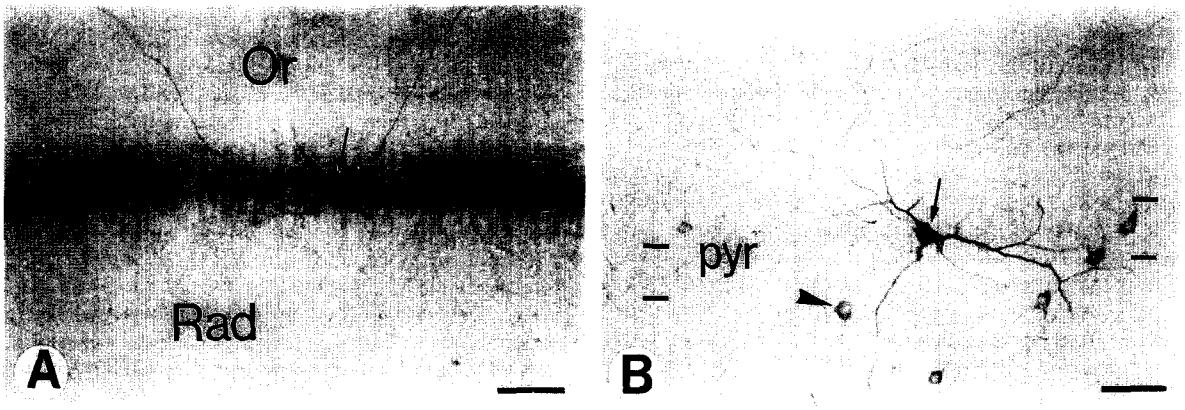

FIG. 3. NADPHd activity in hippocampal CA1 (marked between lines) of brain sections that were mildly fixed (A), that is, with 2% paraformaldehyde, 0.05% glutaraldehyde, or strongly fixed (B), that is, with 2.5% glutaraldehyde postfixation. In both fixations Golgi-like staining is seen of interneurons (arrow). In mildly fixed hippocampus pyramidal cells and dendrites have NADPHd activity (A), which disappears after postfixation with glutaraldehyde (B). Note that this type of outstanding interneuron staining was not visible in the native in-situ blot (see Fig. 2C). Interneurons (arrowhead) in radiate and principal cell layer are faintly visible. These interneurons were in same amounts observed in mildly fixed brains. Bars: A, B = 100 μm. Abbreviations: or, oriens layer; pyr, pyramidal layer; rad, radiate layer.

gyrus (Fig. 3B, arrowhead). Substitution of NADPH by NADH resulted in nonspecific background staining of the entire section.

Distribution in fresh brain sections with and without aldehyde postfixation. Histochemical staining of glass-mounted unfixed brain sections visualized diaphorase activity in a similar pattern as observed in the in situ nitrocellulose blot. No gross differences were observed between NADHd and NADPHd. Therefore, in the unfixed brain these histochemical procedures show a general NBT formazan staining. In the cerebral cortex, most neurons, pyramidal as well as various types of interneurones, showed enzyme activity. Neuronal precipitation in the prefrontal cortex was markedly more intense as compared to that in the parietal cortex. This was especially true for pyramidal neurons in Layer 5. Astrocytes throughout the brain showed a dense reaction. Furthermore, astrocytic endfeet surrounding blood vessels and smooth muscle cells revealed NBT activity. Hippocampal neurons in hippocampal CA1 had a high concentration of enzyme reaction product similar to the nitrocellulose blot. In the cerebellum Purkinje neurons were stained. Postfixation with increasing concentrations of paraformaldehyde caused a complete shift in the staining pattern of NADPHd, whereas the pattern in NADHd was less affected. Very mild paraformaldehyde fixation (0.5%) slightly suppressed the general NBT reactivity in NADPHd histochemistry. In the cerebral cortex a number of nonpyramidal neurons, particularly in the parietal cortex, displayed a higher density of staining than all other cortical cells. Hippocampal neurons in the CA1 cell layer were faintly but still clearly positive. The enzymatic activity in Purkinje neurons was no longer detectable. Increasing concentrations of paraformaldehyde (1, 2, and 4%) gradually produced the distribution pattern of NADPHd as described in many articles: a seemingly random Golgi-like staining of various types of neurons throughout the brain. Whereas the pattern of NADPHd was strongly affected by aldehyde fixation of the tissue, fixation did not alter the general staining pattern of NADHd. It did cause, however, a concentration-dependent shift from clearly cellular localized staining in unfixed brain sections to completely diffuse staining in 4% paraformaldehyde-fixed sections.

NOS Immunoreactivity

Distribution in NC blots. Figure 1C shows the distribution of NOS-I-ir on NC membrane. The cerebellar cortex was among the most densely immunostained regions in native brain tissue with the highest immunoreactivity in the molecular layer followed by that in the granule cell layer. In combination with a restricted resolution in NC membranes it was difficult to study cellular details like staining of basket and granule cells. Lower concentrations of primary antibody (1:200) yielded a staining intensity in which these cell types could be distinguished (Fig. 2E). Surprisingly, part of the Purkinje cell population was also faintly immunopositive, but control experiments with saturation of the antibodies with purified NOS-I showed that the staining in Purkinje cells was unspecific. Other regions with strong NOS immunoreactivity were medial amygdalar regions, hypothalamic areas like the paraventricular and supraoptic nuclei, tectum, and regions in the olfactory bulb. Immunopositive interneurons in cortex and striatum were sparse but very darkly stained (see Fig. 1C). The majority of the interneurons in hippocampal regions were less immunoreactive when compared with the above-mentioned areas, but cells were clearly visible (Fig. 2B). Pyramidal neuronal somata showed faint immunopositivity both in neocortex and hippocampus, which was unspecific staining as indicated by the control experiments. Neuropil staining in cerebral cortex, striatum, and hippocampal dendritic layers was relatively high compared to thalamic areas and overall white matter.

Distribution in perfusion aldehyde-fixed brain sections. The pattern of NOS immunostaining in aldehyde-fixed brain sections was largely similar to the described distribution in native immunoblots and supports the mapping provided in several previous reports [3,19,23]. Although aldehyde fixation strikingly decreased immunoreactivity in the cerebellar cortex at low power observation (Fig. 1C–D), detailed staining of granule, basket, and stellate cells was visible (Fig. 2D). Furthermore, the immunoreactivity in native blots was very strong in brain regions like cerebral cortex and striatum, where individual neurons were easily detectable at low power observation but were more difficult to visualize after aldehyde fixation (Fig. 1D). In fixed brain sections the neurons in these areas also stood out sharply against a clear background, showing a Golgi-like filling with a high resolution of cellular details (Fig. 2A). Control experiments showed that the faint staining in pyramidal and Purkinje neurons was not specific for NOS-I.

DISCUSSION

A clear finding in this study was the similar histochemical staining patterns in the in situ blot after the application of the electron donors NADH and NADPH, making it impossible to separate and identify cells having NADHd, NADPHd, or DT-diaphorase (capable of utilizing either NADH or NADPH as a cofactor for the reduction of NBT) activity in native brain sections. The reliability of the in situ blotting technique for these histochemical stainings was confirmed by the similar results in mounted fresh brain sections. The production of blue NBT formazan in a large number of neurons and astroglial cells in many parts of the unfixed brain, therefore, is catalyzed by unspecified NBT reductase activity. In particular, the results in the mounted fresh brain sections indicate that the precipitation in all of these cells probably has to be considered as specific histochemical staining with a subset of cells in specific brain regions like hippocampal CA1 and cerebellar Purkinje cells demonstrating a higher enzyme activity. Aldehyde fixation on the one hand destroyed the activity of part of these enzymes in many cells, but on the other hand elicited intense, Golgi-like staining of neurons scattered throughout the brain. In this way the tissue fixation completely altered the histochemical staining pattern and produced the NADPHd distribution similar to the well-known distribution of NOS-ir [3,6,11,19,24]. Thus, aldehydes in the fixative apparently inactivate the majority of NBT reductase in the brain. The remainder of the NBT enzyme activity may correspond to the NADPH-dependent reduction of NBT in the crude supernatant fraction of aldehyde-treated tissue homogenate [16]. When mild and strong aldehyde fixation were compared, the sensitivity of NBT reductase activity showed a regional differentiation. Mild fixation annihilated NBT reductases in pyramidal neurons of the cerebral cortex and in cerebellar Purkinje cells, but not in hippocampal CA1 pyramidal cells. High aldehyde concentration removed reductase activity also from the latter neurons, suggesting that in hippocampal pyramidal cells other enzymes or different quantities of enzymes are involved in NBT reductase activity. It was remarkable that after aldehyde perfusion fixation neurons in various brain regions were outstandingly filled with NBT formazan precipitation. Postfixation of fresh mounted brain sections with increasing concentrations of aldehyde showed that in mildly fixed (0.5% paraformaldehyde) brain sections general NBT reductase activity was reduced, whereas a number of neurons began to reach a distinctive density of staining. With increasing concentrations of aldehyde fixation (1, 2 and 4% paraformaldehyde) the general reductase activity disappeared and a number of neurons scattered throughout the brain appeared intensely stained. The intense histochemical staining of seemingly randomly distributed neurons is also characteristic for the NOS-I immunoreactivity in both fixed and unfixed brain. In the unfixed mounted brain tissue as well as in the in situ blots on NC membranes, no cells showing this extremely high NBT reductase activity were found. This increase in NADPHd stain in part of the neurons upon fixation is unexpected because biochemical studies [16] show that paraformaldehyde inactivates most of the NADPHd activity in brain tissue crude supernatant. It remains to be solved why NADPHd activity is increased in part of the neurons after fixation and how the diaphorase activity of NOS is so differentially enhanced by aldehydes.

The demonstration of NOS by means of antibodies raised against cerebellar NOS-I was less affected by aldehyde fixation, although the intensity of the immunoreaction was somewhat reduced in cerebellar cortex and interneurons in cerebral cortex and striatum. Hippocampal pyramidal neurons and cerebellar Purkinje cells are of particular interest with respect to the NOS-ir. In these cells NO has been implicated in synaptic plasticity phenomena like long-term potentiation (LTP) in the hippocampus [1,2] and long-term synaptic depression (LTD) in the cerebellum [21]. In these processes NO is suggested to act as a possible retrograde messenger, carrying information from postsynaptic elements (e.g., CA1 pyramidal neurons and cerebellar Purkinje cells) to presynaptic terminals [see ref. 24]. However, most studies reported no detectable NOS in these neurons, either with in situ hybridization techniques showing mRNA for NOS [5] or with immunocytochemistry [3,19,23]. Although a faint immunoprecipitation was visible in hippocampal pyramidal cells in fixed as well as in unfixed brain (see Fig. 2A,B and 2D,E), control experiments in which the primary antibody was saturated with purified NOS-I showed that this immunolabeling was due to unspecific binding of anti NOS-I serum. Therefore, we were unable to confirm the recent findings of Wendland et al. [26], who described the existence of NOS-I in hippocampal pyramidal cells. Our results concerning the NOS-ir in both native NC blots and aldehyde-fixed brains showed very dense labeling in various types of neurons, basket cells, and granular cells. In the in situ blot the cellular localization of NOS was already visible at low power observation, which probably is caused by spreading of the reaction product. Because likewise spreading does not generally occur in immunocytochemical staining procedures in the blotting technique, it can be assumed that the amount of native enzyme contained by these immunopositive neurons is very large. This high amount of protein is confirmed by the high density of histochemical reaction product in aldehyde-fixed brain sections, resulting also in visibility of neurons at low power observation (see Fig. 1B). The results in fresh mounted brain sections with and without paraformaldehyde postfixation indicated that aldehydes are responsible for the increase in the amount of enzyme in part of the neurons.

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